Three-dimensional Spheroid Culture Combined with Hypoxic Preconditioning Can Promote the Proliferation and Osteogenic Differetiation of BMSCs in Low-oxygen Environment in Vitro

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Abstract

Background: Presently, it is difficult to use BMSCs in bone regeneration because the cells are severely weakened or even die after transplantation into the body after *in vitro* culture. This is due to the difference between the living environment of cells cultured *in vitro* and *in vivo*. This study explored a fast and efficient method for developing BMSCs with stronger survival and osteogenesis ability in a harsh environment through hypoxic preconditioning and three-dimensional (3D) culture to transform BMSCs into cell therapy.

Methods: BMSCs were treated with various hypoxic preconditioning processes *in vitro*, including sustained hypoxia, intermittent hypoxia, and sustained normoxia, in planar culture and 3D spheroid culture. Cell Counting Kit-8 assay, apoptosis determination assay, and live-dead staining were used to evaluate the proliferation and survival abilities of BMSCs. Alkaline phosphatase, alizarin red staining, and osteogenic-related proteins were used to assess the osteogenic differentiation abilities of BMSCs. Meanwhile, the proliferation, survival, and osteogenesis abilities of BMSCs between planar culture and 3D spheroid culture were compared.

Results: BMSCs showed stronger proliferation and survival abilities after intermittent hypoxic preconditioning and 3D spheroid culture. Moreover, cells cultured based on intermittent hypoxic preconditioning and 3D spheroid culture showed less apoptosis and higher osteogenic differentiation abilities than those cultured via conventional treatment in the hypoxic microenvironment simulated by matrix gel placed in a three-gas incubator, including alkaline phosphatase activity, cell calcium content, and expression of osteogenic-related molecules.

Conclusions: Intermittent hypoxic preconditioning and 3D spheroids culture at specific stages of BMSCs significantly increase the proliferation, survival, and osteogenic differentiation abilities of BMSCs, thus improving the application potential of seed cells in bone regeneration.

1 Introduction

The repair of severe bone injuries caused by severe trauma, tumor resection, and congenital malformations, especially severe bone defects, is still facing great challenges in the clinical practice of oral and maxillofacial surgery. BMSCs, as seed cells, have been effectively used to treat bone defects in recent years. BMSCs can differentiate into osteoblasts, thus promoting bone tissue regeneration and repair *in vivo*. BMSCs can also differentiate into vascular endothelial cells and secrete various angiogenic factors, thus improving the blood supply to the bone defect area, enhancing bone defect repair [1–4]. However, these functions are usually gradually lost after *in vitro* culture, mainly because the *in vitro* culture environment is a non-physiological monolayer [5–7]. The sudden ischemia and hypoxia environment caused by the lack of a microvascular network often results in the mass death of seed cells when BMSCs are transplanted into the bone defect area *in vivo* [8, 9]. Therefore, it is necessary to improve the current culture methods of BMSCs to effectively improve the survival and differentiation abilities of

cells in the ischemic and hypoxic environments by providing a solution to the cell survival and function maintenance issues during the transformation of BMSCs cell therapy into clinical application.

Conventional *in vitro* culture is commonly used in a relatively high oxygen (21% O_2) culture environment. Oxygen concentration in the local bone defect area is about 1% or lower [10–12]. The difference in partial oxygen pressure between the *in vivo* and *in vitro* cultures can change the biological behavior of cells before and after transplantation, thus significantly impacting the transplantation. Previous studies have found that BMSCs cultured in the normoxic environment have stronger biological activity and improved ability to repair critical bone defects after 48 h of pretreatment with hypoxia before being placed in hypoxic environment [13]. The previous studies cultured all the BMSCs in conventional conditions (21% O_2, 37℃, 5% CO_2) and brought them up to the 3rd passages. This culture condition instantly and significantly alters the environment in which BMSCs live, significantly different from the environment in which they grow *in vivo*. As a result, their biological behaviors are altered. Therefore, BMSCs may maintain their biological behavior, reduce their mortality after transplantation, maintain their stemness, and accelerate their proliferation when cultured at 37 ℃, 1% O_2, and 5% CO_2, thus providing an efficient method for obtaining and applying BMSCs. This approach may further improve the application efficiency of BMSCs in the transplantation of bone defects.

Most methods in previous studies are limited to the non-physiological monolayer culture. Although BMSCs have been rapidly amplified in non-physiological monolayer culture, apoptosis still occurs after transplantation [14–18]. However, the implantation has reduced apoptosis and longer survival when the cells are interlinked to form an aggregate than the equivalent number of free cells [19]. BMSCs microspheres are multi-cell aggregates formed under 3D culture conditions. BMSCs microspheres have stronger viability, angiogenesis, and osteogenesis than two-dimensional (2D) monolayers in the ischemic and hypoxic environment due to the promotion of the interaction between the cells. Additionally, the formation of BMSC spheroids can effectively up-regulate growth factors, including vascular endothelial growth factor and angiogenin, thus greatly improving the angiogenic potential of BMSCs and further improving their survival rate after transplantation [20–22].

In this study, a rapid and efficient method for obtaining BMSCs with stronger proliferation, osteogenesis, and survivability in harsh environments was explored using hypoxic preconditioning and the formation of BMSCs spheroids. This approach may provide a solution to the cell survival and functional maintenance of BMSCs in the transformation of cell therapy into clinical use.

2 Materials And Methods

2.1 Isolation and culture of BMSCs

BMSCs were obtained from femur bone marrow and tibia of 4-week-old SD rats. The BMSCs were then divided into three groups from primary generation: continuous hypoxic culture (1% O_2, 37 ℃, 5% CO_2), interrupted hypoxic culture (primary cells transferred to normoxic culture after hypoxic culture for 48 h),

continuous normoxic culture (21% O_2, 37 ℃, 5% CO_2). The culture medium was replaced every two days. Subculture was conducted when the cell growth density reached 90%, then the cell morphology and growth were closely observed. The cells were cultured to the third generation for future experiments. The 3rd generation BMSCs were divided into four groups (Table 1) after 48 h of hypoxic preconditioning and re-hypoxic preconditioning at 48–120 h of culture to analyze whether intermittent hypoxic preconditioning combined with 3D culture can synergically enhance survival and osteogenic activities of BMSCs *in vitro*.

Table 1
Group and culture condition

	I	II	III	IV
	2D normoxia	2D hypoxia-normoxia	3D normoxia	3D hypoxia-normoxia
0–48 h	21%O_2	21%O_2	21%O_2	21%O_2
48–120 h	21%O_2	1%O_2	21%O_2	1%O_2

2.2 Proliferation curve and CCK8 assay

The 3rd passages of BMSCs were uniformly inoculated into 96-well plates at a density of 5×10^4/mL and transferred to hypoxic culture to construct the proliferation curves of the three groups of BMSC under different hypoxic conditions. The proliferation ability of BMSCs in each group was detected at five time points (0, 1, 2, 3, and 4 days) using CCK-8 assay kit. Briefly, the supernatant in each well was discarded, then 10 μL CCK-8 reaction solution and 100 μL medium mixture were added to each well under dark conditions and incubated for 2 h. A microplate reader was used to detect the OD value of each group at 450 nm. The mean value was used as the experimental result, and the cell proliferation ability curve was drawn.

2.3 Live-dead staining

The third generation of BMSCs was evenly inoculated into 96-well plates at a density of 5×10^4/mL and transferred to hypoxia culture. The live-dead cell viability test kits were used to determine survival of BMSCs in each group under hypoxia conditions at 24 h and 48 h. Briefly, the culture medium in the well plate was discarded, then the plates were rinsed thrice using PBS buffer solution. Live-dead solution (0.5 ml) was then added to each well, and the cells were immersed. The staining solution was removed at room temperature for 45 minutes to terminate the incubation. Percentages of live and dead cells were calculated based on fluorescence readings at 530 nm and 600 nm.

2.4 Generation of BMSCs spheroids via the Insphero hanging drops technique

The hanging drops technique was used to generate BMSCs spheroids based on Insphero (InSphero AG) manufacturer's instructions. The third generation of BMSCs, pretreated with 1% hypoxia for 48 h, was divided into three groups based on inoculation densities of 2.4×10^4, 4.8×10^4, and 9.6×10^4 per well to construct BMSCs microspheres. BMSCs cell suspension (40 μL) was then added to the top of GravityPLUS™ automatic suspension plate to form suspension droplets. The cells in the suspension droplets slowly moved and gathered at the bottom of the suspension droplets, then proliferated to form 3D microspheres. The BMSCs microspheres were formed after 48 h of conventional culture. An excessive amount of substrate was added to the upper end once to make the suspended droplets containing BMSCs microspheres fall into the GravityTRAP™ plate. GravityTRAP™ board's proprietary non-adhesive features allow BMSCs microspheres to be cultured for several weeks without attachment and aggregation. Moreover, its unique design enables substrate replacement without disturbing microstructures, and thus imaging analysis can be performed directly through the culture plate.

2.5 Apoptosis determination assay

The apoptosis activity of caspase was determined in two parts. Caspase-Glo reagent (100 ul) was added to each A96-well plate. The contents of each well were then mixed at 300–500 rpm for about 30 seconds on a shaking machine. The samples were incubated at room temperature for 3 h, then the fluorescence value of each sample was measured using a fluorescence luminescence instrument. Nucleic acid quantitative analysis reagent (100 ul) was added into each B96-well plate and allowed to react for 5 minutes. The DNA content of the sample was determined using a fluorescence luminescence instrument.

2.6 Alkaline phosphatase and alizarin red staining

The P3 generation BMSCs pretreated for 48 h were divided into four groups (Table 2) for subsequent determination of osteogenic indicators to explore the effect of intermittent hypoxic preconditioning on the osteogenic activity of 3D BMSCs microspheres. The supernatant of the cells on the 3rd, 5th, and 7th days of osteogenic induction was discarded, and the cells were washed twice with PBS. Alkaline phosphatase dye (0.5 mL) was added to each well, allowed to stand for 1 minute, then the dye was discarded, and the cells were washed twice with PBS. An inverted microscope was used to visualize the stained cells. Cell lysates were collected after seven days of osteogenesis induction. The absorbance was then measured at 405 nm. The supernatant of the cells was discarded after the 7th, 14th, and 21st days of osteogenic induction, and the residual medium was removed by washing thrice with PBS buffer. The residual medium was fixed with 4% paraformaldehyde solution for 10 minutes. The residual paraformaldehyde solution was removed by washing thrice using PBS buffer. Alizarin red staining was then performed, and an inverted microscope was used for visualization.

Table 2
Group and culture condition

	I	II	III	IV
	2D normoxia	2D hypoxia-normoxia	3D normoxia	3D hypoxia- normoxia
0–48 h	21% O_2 Matrigel	21% O_2 Matrigel $_2$	21% O_2 hanging-drop culture plate	21% O_2 hanging-drop culture plate
48–96 h	21% O_2 Matrigel	1% O_2 Matrigel	21% O_2 low-adherence culture plate Matrigel	1% O_2 low-adherence culture plate Matrigel
96 h-21 d	21% O_2 osteogenic induction	21% O_2 osteogenic induction	21% O_2 osteogenic induction	21% O_2 osteogenic induction

2.7 Cell calcium content

The cells were collected on the 7th, 14th, and 21st days of osteogenesis induction. The matrix glue containing cells was collected in the lysate and divided into two groups in two 96-well plates. The A96-well plate was centrifuged in a low-speed centrifuge, and the supernatant was taken as the sample. The pre-prepared calcium chromogenic working solution (2 ml) was added to 25 ul of sample in a dry test tube, mixed well, then allowed to stand at room temperature for 5 minutes. The absorbance was detected at 575 nm. Nucleic acid quantitative analysis reagent (100 ul) was added to each B96-well plate, then allowed to react for 5 minutes. The DNA content of the sample was determined using a fluorescence luminescence instrument.

2.8 Western blot analysis

The contents of Osteopotin and Runx2 were detected using western blot after 21 days of osteogenesis induction. Precooled cell lysate (800 μL) was then added to each well plate. Total cell protein was extracted after cell lysates were fully lysed. Protein concentration was measured via the BCA method. Protein electrophoresis was also performed. The membrane was transformed, sealed, and incubated with primary antibody at room temperature for 30 minutes, then transferred to low temperature overnight. The membrane was then incubated with a secondary antibody for 2 h and developed using bio-RAD chemiluminescence imager.

2.9 Statistical Analysis

Data are expressed as mean ± standard deviation. Data analysis was performed using a one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test using GraphPad Prism 9.0 software. $P < 0.05$ was considered a significant level.

3 Results

3.1 Hypoxic preconditioning for 48 h improves BMSC survival and proliferation

The morphology of primary and P3 generations of BMSCs were observed to investigate the effects of different hypoxia pretreatment conditions on the survival and proliferation ability of non-physiological monolingual BMSCs *in vitro*(Fig. 1a). Moreover, the time interval of subpassage under three different hypoxic pretreatments was analyzed (Fig. 1b). The subculture time was significantly shorter in the hypoxia-normoxia group than in the hypoxia and normoxia groups（$P < 0.0001$, $n = 6$）, indicating the passage time of BMSCs after 48 h of hypoxic pretreatment is shorter and the culture efficiency is higher. The P3 generation of BMSCs in each group was cultured in a hypoxic environment after pretreatment, and then the cell proliferation ability curve was plotted (Fig. 1c). The proliferation ability of cells in the hypoxia group gradually increased with culturing days after exposure to 1% hypoxia. The proliferation ability of cells in the hypoxia-normoxia and normoxia groups decreased and then increased after exposure to the 1% O2, reaching the lowest point at 48 h(Fig. 1d). Moreover, the proliferation ability of cells in the hypoxia-normoxia group reduced after exposure to the hypoxia environment compared with that of the cells in the normoxia group. However, the proliferation ability of the cells in the hypoxia-normoxia group recovered faster after 48 h than that of cells in the normoxia group. It exceeded the proliferation ability of cells in the hypoxia group on the 3rd day. These results indicate that continuous hypoxic culture and hypoxic culture for 48 h can improve the proliferation ability of primary BMSCs in hypoxic environment. Cell survival was detected by staining live and dead cells (Fig. 1e, f). Several BMSCs in the hypoxia-normoxia and normoxia groups died under the hypoxia environment. However, the cell survival was higher in the hypoxia-normoxia group than in the normoxia group, indicating that BMSCs in the hypoxia-normoxia group had a stronger survival ability than those in the normoxia group under the hypoxia environment. Moreover, cells in the hypoxia group had a very strong survival ability under the hypoxia environment, significantly higher than that in the other two groups at 48 h ($P < 0.0001$, $n = 6$), and thus could significantly proliferate.

3.2 Hypoxic preconditioning for 48 h stimulates BMSCs osteogenesis differentiation

Alkaline phosphatase staining was performed after osteogenic induction of the third generation of pretreated BMSCs in each group (Fig. 2a, b). The alkaline phosphatase activity of BMSCs in the hypoxia-normoxia and normoxia groups gradually increased. In contrast, the alkaline phosphatase activity of BMSCs in the hypoxia group slightly decreased. Moreover, the alkaline phosphatase activity of BMSCs in the hypoxia-normoxia group was significantly higher than that of BMSC in the hypoxia and normoxia

groups after seven days of osteogenic induction. Alizarin red staining was also performed after osteogenic induction of the third generation of pretreated BMSCS in each group (Fig. 2c). Mineralized nodules gradually increased with induction time after osteogenic induction of BMSC in the hypoxia-normoxia and normoxia groups. Few nodules were mineralized after hypoxia bone induction. The number of mineralized nodules was significantly higher in the hypoxia-normoxia and normoxia groups than in the hypoxia group after 14 and 21 days of osteogenesis induction, especially in the hypoxia-normoxia group. These results indicate that 1% continuous hypoxic culture can inhibit the osteogenic activity of BMSC, while 1% hypoxic pretreatment for 48 h can enhance the osteogenic activity of BMSC.

3.3 Generation of BMSCs spheroids *in vitro*

The 3D BMSCs microspheres were constructed with three different inoculation densities (Fig. 3a, b) using Insphero suspended drop culture technology to explore the effect of cell inoculation density on the growth of BMSCs microspheres and the optimal *in vitro* culture density of BMSCs microspheres. Visible BMSC microspheres with different diameters were constructed using different inoculation densities (Fig. 3c). BMSCs microspheres were observed under a microscope after 48 h of culture, and their diameters were measured. The diameters of BMSCs microspheres were proportional to the inoculation densities (Fig. 3d). The higher the inoculation density, up to a certain inoculation density, the larger the sphere diameter. However, the sphere diameter and cell inoculation density did not show a multiple growth relationship.

3.4 Comparison of proliferation and viability of BMSCs spheroids with different diameters under hypoxic condition

The BMSCs microspheres were cultured in a low oxygen environment (1% O_2, 37°C, 5% CO_2). The apoptotic activity was then measured to compare the proliferation and survival ability of BMSCs with different diameters(Fig. 4a). Spheres with 4.8×10^4 and 9.6×10^4 cell densities had greater apoptosis than that with 2.4×10^4 cell density ($P < 0.0001$, $n = 12$), indicating that the BMSC microspheres with 2.4×10^4 cell density had a stronger proliferation ability than those with 4.8×10^4 and 9.6×10^4 cell densities. Compared with cell number in hypoxic environment ($P < 0.0001$, $n = 12$, Fig. 4b, c), cell death was higher after exposure to 1% hypoxic culture for 24 h. The dead cells were mainly concentrated in the core of the spheres. The staining results of live-or dead cells and the percentage of dead cells cultured in a 1% hypoxic environment for 24 h and 48 h in each group were also compared. Several BMSCs microspheres in the 9.6×10^4 group died after 24 h under hypoxic environment. The number of dead cells decreased after 48 h. Cell death was significantly lower in the 2.4×10^4 group than in the 4.8×10^4 and 9.6×10^4 groups after 1% hypoxic culture for 24 h and 48 h, suggesting that the survival rate was higher in the 2.4×10^4 group than in the 4.8×10^4 and 9.6×10^4 groups.

3.5 The survival and osteogenic activity of BMSCs microspheres increase under hypoxic condition after intermittent hypoxic preconditioning

Previous sections (3.1–3.2) showed that the proliferation, survival, and osteogenic ability of BMSCs were significantly enhanced after 48 h of hypoxia pretreatment *in vitro*. Sections 3.3–3.4 showed the optimal inoculation density (2.4×10^4) for generating 3D BMSCs microspheres. BMSCs pretreated with hypoxia for 48 h were divided into four groups (Table 2), and caspase activity was determined. A comprehensive comparison of the apoptosis curves of the four groups showed that the 2D cultured BMSCs had higher apoptotic activity than the BMSC microspheres, indicating that BMSC microspheres had stronger proliferation activity than the 2D cultured BMSCs ($P < 0.0001$, $n = 6$, Fig. 5a). The apoptosis activity of 3D cultured BMSC microspheres after hypoxic pretreatment for 48 h was significantly lower than that of 3D cultured BMSCs microspheres without 1% hypoxic pretreatment($P < 0.0001$, $n = 6$). This suggests that intermittent hypoxic pretreatment can reduce the apoptotic activity of BMSCs microspheres and improve their survival and proliferation activities.

Alkaline phosphatase staining was performed after osteogenic induction of BMSCs based on the conditions shown in Table 2 (Fig. 5b). The ALP levels in each group were time-dependent. The activity of ALP gradually increased with increasing osteogenic induction time. For instance, ALP content was not significantly different among all groups at days 0–3. However, ALP content was significantly different among all groups at days 5–7 ($P < 0.0001$, $n = 6$). ALP contents after 5–7 days of osteogenic induction were highest in the 3D hypoxia-normoxia, followed by 3D normoxia, 2D hypoxia-normoxia group, and lowest in 2D normoxia. Intracellular calcium concentration in each group was also determined (Fig. 5c). The calcium content in each group was time-dependent. The calcium content gradually increased with increasing osteogenic induction time. For instance, calcium content was different among all groups at day 7. Moreover, the calcium content of each group significantly increased at 7–14 days and 14–21 days ($P < 0.0001$, $n = 6$). The osteogenic calcium contents were higher in the 3D hypoxia-normoxia group, followed by the 3D normoxia group, 2D hypoxia-normoxia group, and lowest in the 2 D normoxia group. Western blot was used to detect the content of osteogenic-related proteins at day 21 of osteogenic induction (Fig. 5d, e, f). The expressions of Osteopotin and Runx2 in each group were highest in the 3D hypoxia-normoxia group, followed by 3D normoxia, 2D hypoxia-normoxia, and lowest in the 2D normoxia group, consistent with the quantitative determination of calcium and alkaline phosphatase activities. These results indicate that BMSCs pretreated with intermittent hypoxic have stronger osteogenic ability under hypoxia environment.

4 Discussion

Proper pretreatment of BMSCs before *in vivo* transplantation enhances its proliferation, survival, and osteogenic activity and is thus essential for the further clinical application of BMSCs [22, 23]. This study provides an *in vitro* pretreatment method for BMSCs(combination of interrupted hypoxia pretreatment and three-dimensional culture) for efficient transplantation *in vivo*.

The cell behaviors change during the *in vitro* culture due to the significant changes in the growth environment[24, 25]. Moreover, only a few primary MSCs can be directly obtained from the body. However, a sufficient number of cells can be obtained by generating 3D culture microspheres after a certain period

of *in vitro* culture [26, 27]. Herein, although the cells were cultured in a non-physiological monolayer during cell amplification, hypoxic pretreatment was used to simulate the ischemia and hypoxia environments *in vivo* a. As a result, cells could survive in an anoxic environment, thus ensuring that the cells maintain amplification and proliferate and survive in an anoxic environment. However, the adverse effects of hypoxia on cells were not ignored. The results showed that continuous hypoxia had a certain inhibitory effect on the osteogenic differentiation of BMSCs. Therefore, the effects of different hypoxic pretreatment methods on cells were analyzed, the traditional hypoxic culture method was optimized, and finally the intermittent hypoxic culture method was selected for further analysis. BMSCs could proliferate and survive in an anoxic environment and maintain osteogenic differentiation to the maximum extent under the intermittent hypoxic culture method. Moreover, all aspects of biological properties of cells could be balanced, thus suitable in subsequent application.

The 3D cell culture technology can simulate the physiological environment of cells in organisms and maintain the contact between cells. Therefore, it is more suitable for maintaining biological behavior of isolated cells. Currently, 3D cell culture techniques are grouped into two main categories: those with and without scaffolds [28]. The 3D cell culture techniques with scaffolds, such as hydrogel technology, mainly rely on the support and guidance of natural or artificial scaffolds to enable cell growth on scaffolds and are widely used at present [29]. The 3D cell culture techniques without scaffolds mainly include suspension drop technology, suspension technology, and ultra-low adhesion surface. Stent-free-3D cell culture technology mainly uses a specific culture plate to suspend and aggregate cells into a 3D sphere [30, 31]. Compared with 3D cell culture technology with scaffolds, 3D cell culture without scaffolds can obtain a single cell sphere without dealing with cell scaffolds. Moreover, the volume and number of cell spheres are easier to control when using 3D cell culture without scaffolds. Herein, cell suspension was added above the hole of GravityPLUS™ automatic suspension plate. The geometry of the hole plate guides the cells and culture medium through the hole to form a stable suspension. Each hole forms a droplet, and each droplet contains a sphere whose size can be controlled by the density of the inoculated cells. GravityPLUS™ automatic hanging drop board can be sterilized and reused, thus significantly saving costs. As a result, this stent-free 3D cell suspension culture technique was used in the *in vitro* cell experiments.

Although the 3D culture represented by microspheres simulates the 3D growth environment of cells *in vivo*, the influence of large microspheres on cell apoptosis cannot be ignored. The diameter of microspheres affects plasma osmotic pressure, oxygen content, and nutrient supply in cell culture microspheres [32]. In this study, the cells at the center of the microspheres rapidly underwent apoptosis in a short period when the diameter of BMSCs microspheres was too large. Although increasing the inoculation density can successfully generate microspheres with a larger volume, it does not necessarily indicate that the cells can maintain a high survival rate. Therefore, BMSCs microspheres should have a certain range of diameter to improve good cell performance and efficiency of cell application. Moreover, waste resources are minimized, thus obtaining a good practical application and improving the clinical application potential of BMSCs, such as seed cells.

5 Conclusions

In conclusion, this study showed that *in vitro* intermittent hypoxic preconditioning combined with 3D spheroid culture can promote the proliferation, survival, and osteogenesis of BMSCs under hypoxic conditions at appropriate culture concentration and hypoxic conditions. Therefore, these findings may provide a new strategy for improving the function of BMSCs before transplantation into the body.

Abbreviations

BMSCs Bone marrow mesenchymal stem cells

3D Three-dimensional

2D Two-dimensional

DNA Deoxyribonucleic acid

Ca Calcium

ALP Alkaline phosphatase

PBS Phosphate buffer saline

CCK-8 Cell counting kit-8

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

MEM Minimum essential medium

FBS Fetal bovine serum

RUNX2 Runt-related transcription factor 2

Declarations

Ethics approval and consent to participate

This research was approved by the Ethics Committee of West China College of Stomatology, Sichuan University, China(WCHSIRB-D-2016-206).

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The specific contributions of the authors are as follows. (1) XQ, ZF and WJ contributed to the conception and design of the study; (2) GL, RM, ZT, JT and BY acquired, analyzed and interpreted the data; (3) XQ and ZF were the main writers of the manuscript, contributed equally to this work and should be considered co-first authors; (4) XQ, ZF and WJ were responsible for the critical revision of the manuscript. All authors have read and approved the final manuscript.

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Figures

Figure 1

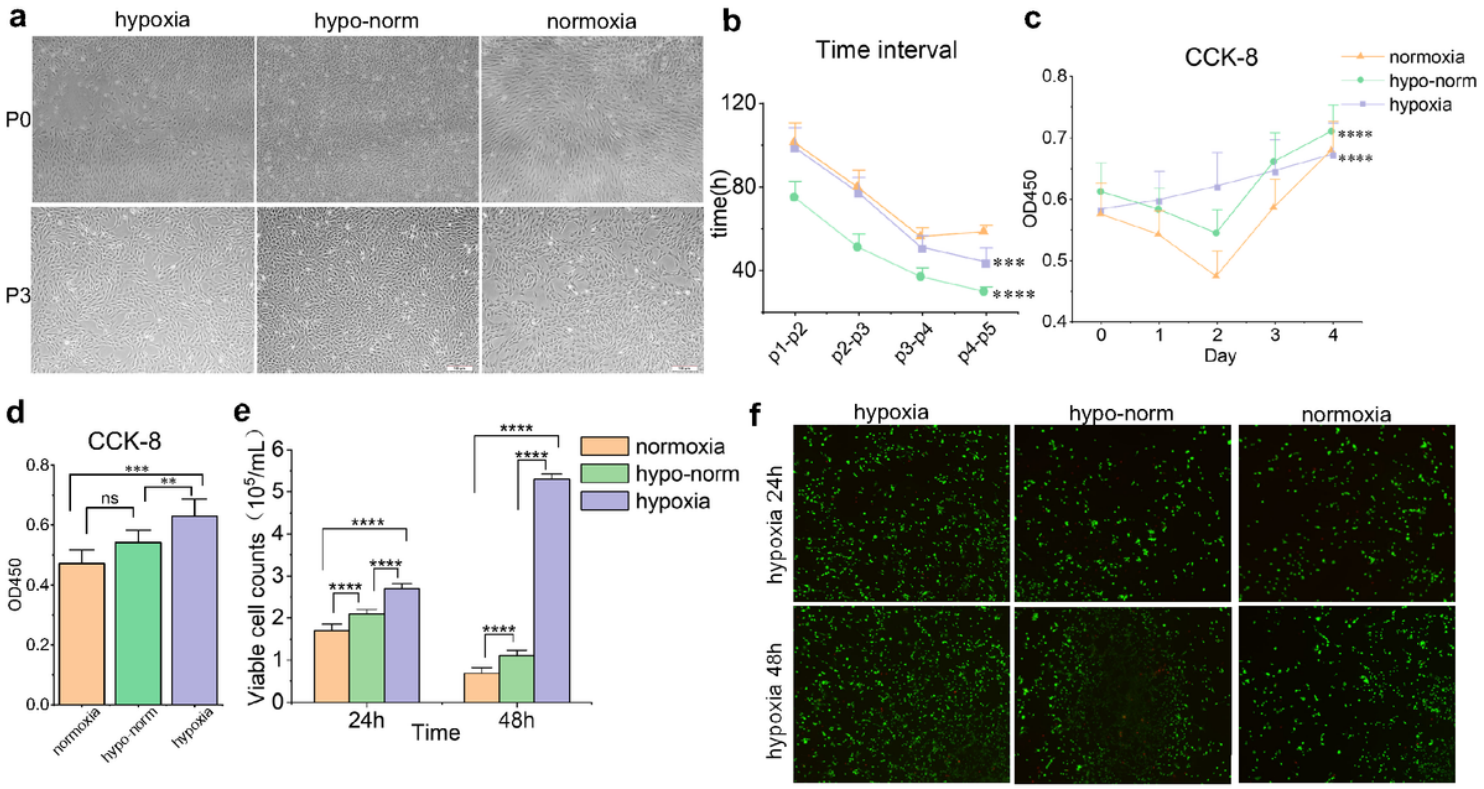

Figure 1

Hypoxic preconditioning for 48 h improves BMSCs survival and proliferation.

a Morphology of BMSCs in 0th and 3rd passages under different hypoxia preconditioning. **b** Cell passage interval time. **c** CCK8 assay results of 3rd passages of BMSCs under hypoxia condition. **d** CCK8 assay results of 3rd passages of BMSC under hypoxia condition for 48 h. **e** Viable cell counts after 24 h and 48 h under hypoxia condition. **f** Live or dead cells stained with FDA (live, green) and PI (dead, red). Results are presented as the mean ± SEM (n = 6). Analysis of variance (ANOVA) was used determine statistical significance (**p<0.01, ***p<0.001, ****p<0.0001)

Figure 2

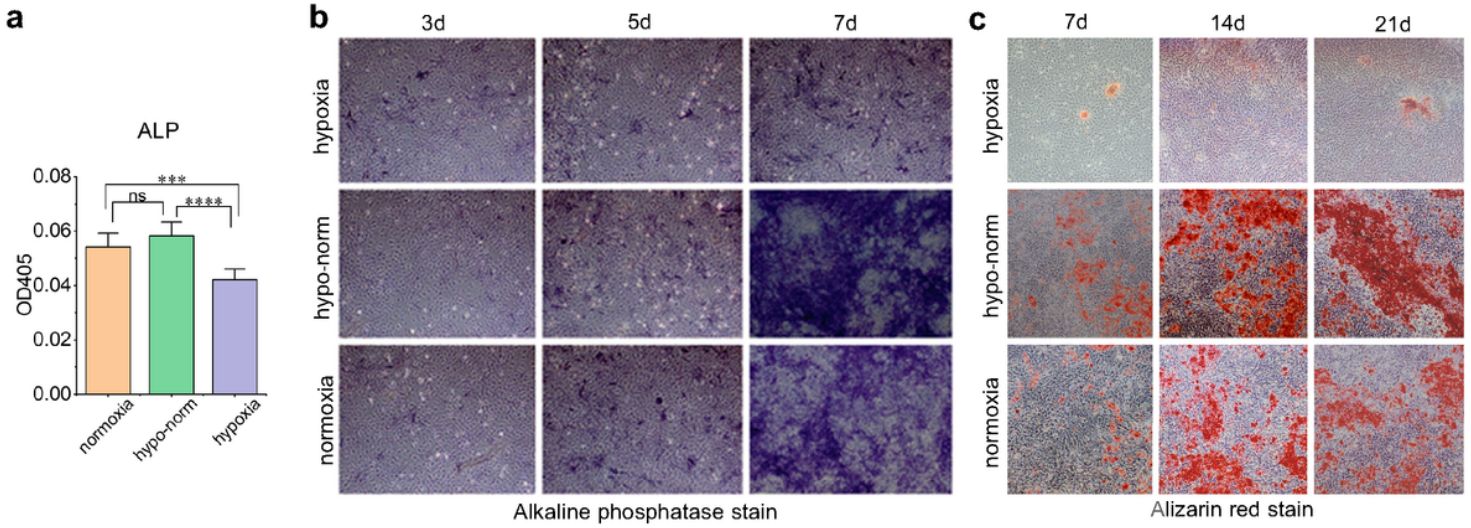

Figure 2

Hypoxic preconditioning for 48 h stimulates BMSCs osteogenesis differentiation.

a ALP activity after osteogenic induction for seven days. (***p<0.001, ****p<0.0001). **b** ALP activity of BMSCs after osteogenic induction for 3, 5, 7 days. **c** Alizarin red staining after osteogenic induction for 7, 14, 21 days.

Figure 3

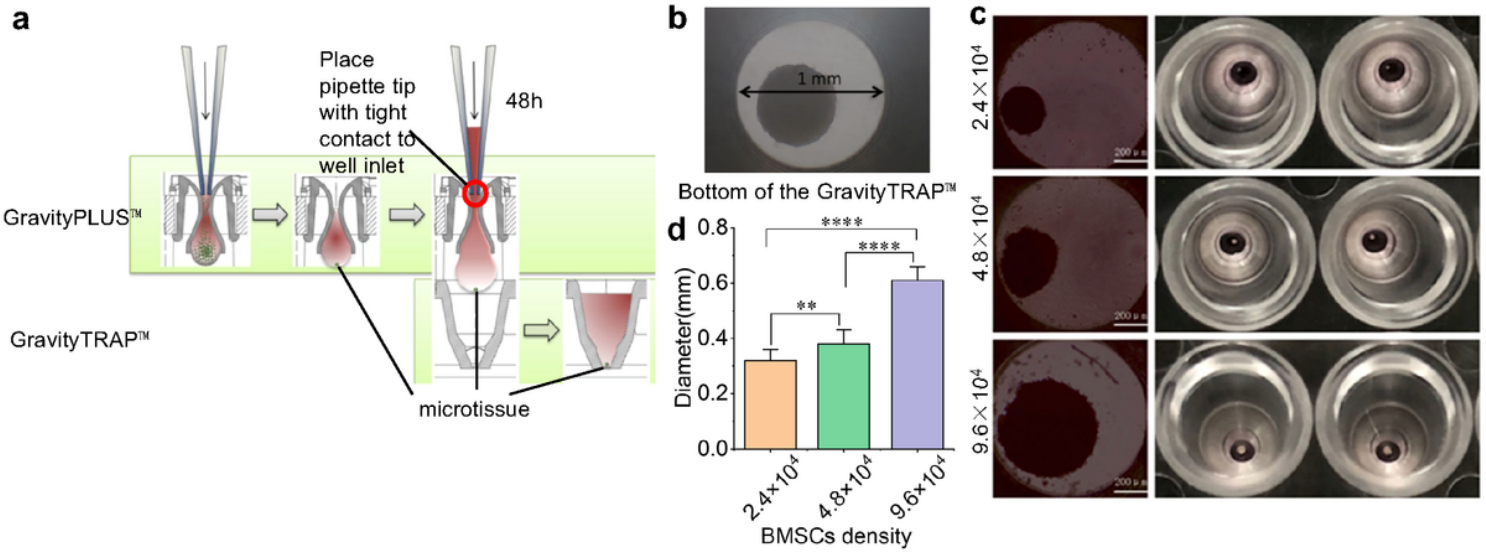

Figure 3

Successfully generated BMSCs spheroids *in vitro*.

a Generation of spheroids based on Insphero (InSphero AG) manufacturer's instructions. **b** One BMSC spheroid per macrowell. **c** Visible BMSC spheroids generated at low, medium, and high inoculation densities after 48 h of culture (Scale bar = 200μm). **d** The diameter of BMSCs spheroids. (**p<0.01, ***p<0.001, ****p<0.0001)

Figure 4

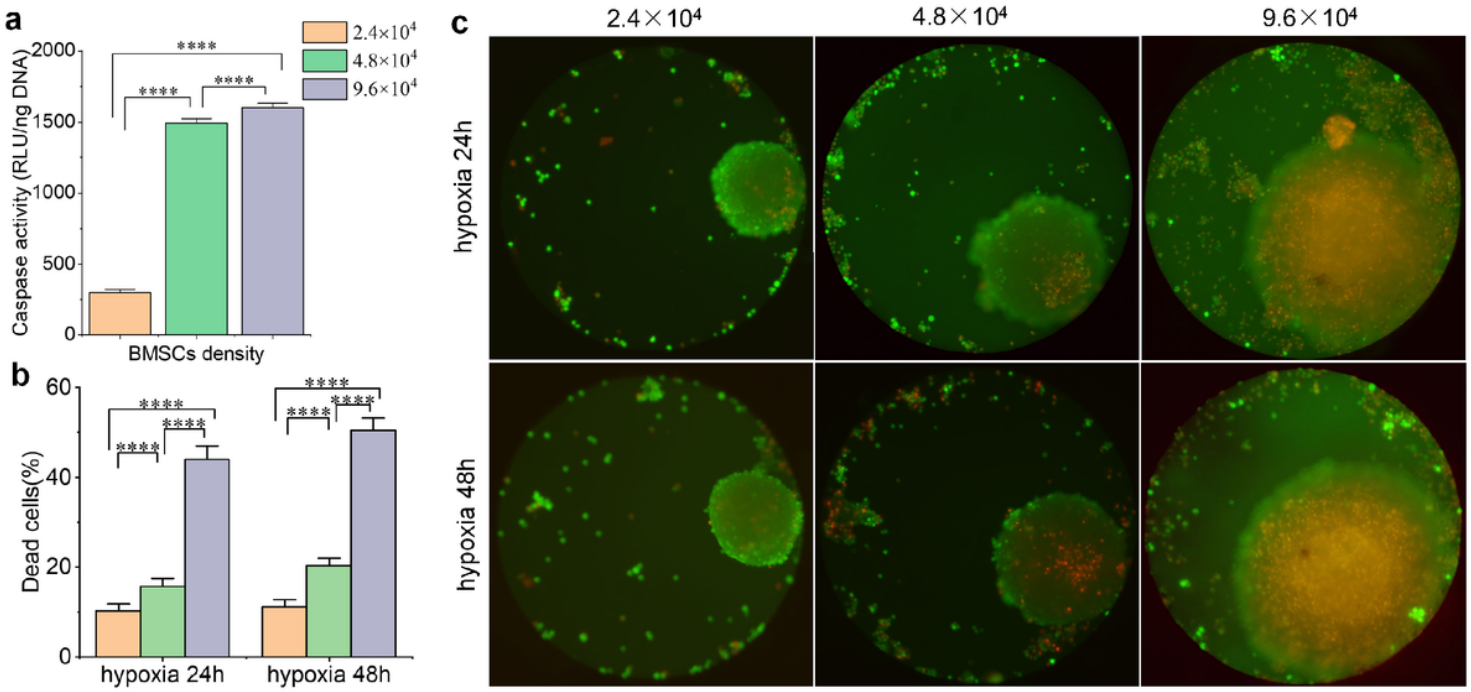

Figure 4

Comparison of proliferation and viability of BMSCs spheroids with different diameters under hypoxic condition.

a Caspase activity determination. **b** The percentage of dead cells after 24 h and 48 h under hypoxic condition. (****p<0.0001) **c** Live or dead cells stained with FDA (live, green) and PI (dead, red).

Figure 5

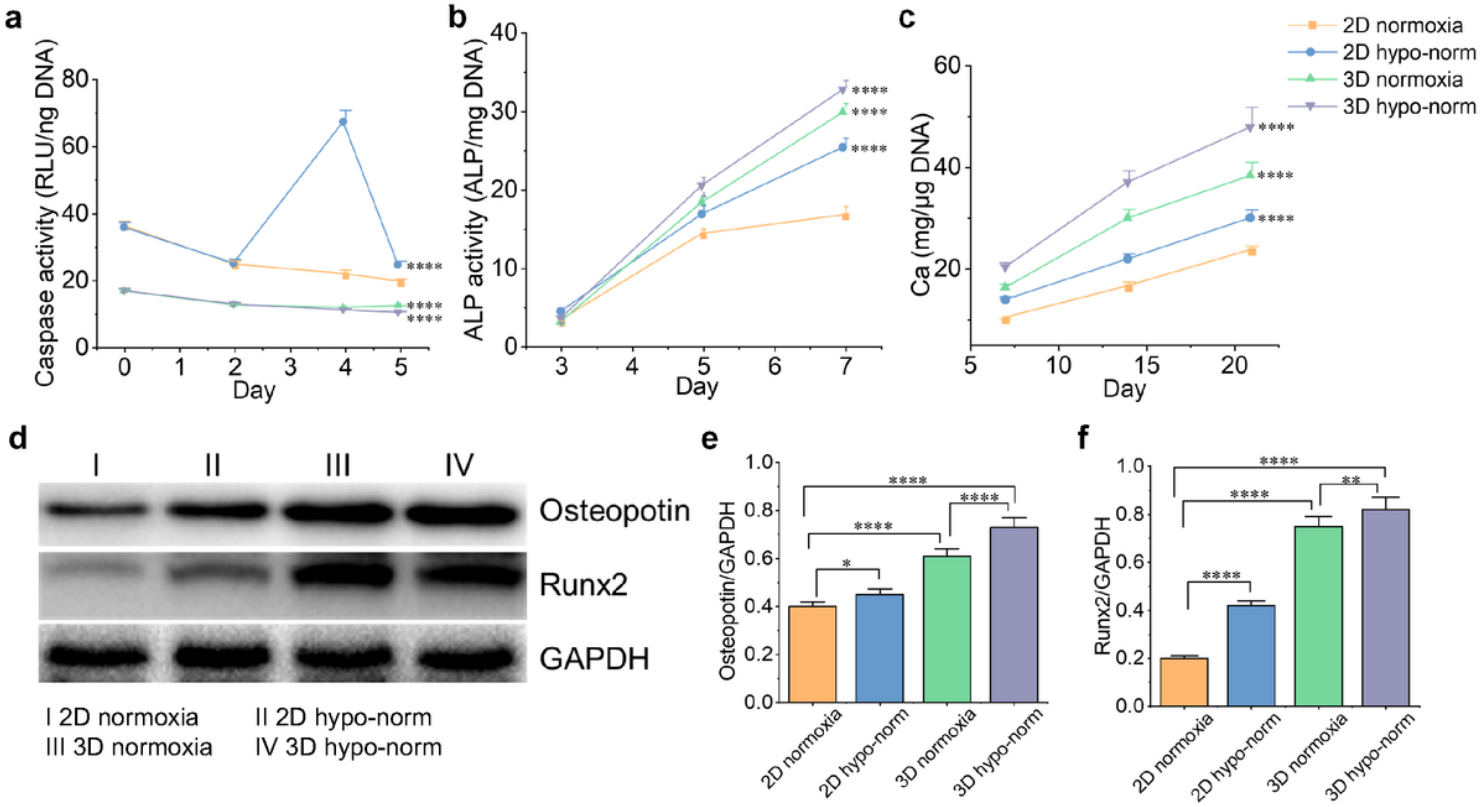

Figure 5

The survival and osteogenic activities of BMSCs microspheres increase under hypoxic conditions after intermittent hypoxic preconditioning.

a Caspase activity after osteogenic induction for 0, 2, 4, 5days. **b** ALP activity after osteogenic induction for 3, 5, 7days. **c** Cell calcium content after osteogenic induction for 7, 14, 21days. **d** Western blot. **e** Expression of osteoprotin. **f** Expression of Runx2. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)